Influence of Tumor Microenvironment Biophysical Properties on Myoferlin-Mediated Changes in Breast Cancer Cell Migration and Invasion

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Abstract

The five-year survival rate for breast cancer decreases from 98.6% to 25.9% if the cancer metastasizes to invade other body tissues (Morris, 2015). Metastasis occurs when cancerous cells migrate away from the original tumor to other parts of the body. This migration requires increased cell motility and previous studies indicate that loss of a membrane repair protein, Myoferlin (MYOF), can be used to reduce the motility of a highly invasive line of breast cancer cells, MDA-MB-231 (Li, 2012). In addition, biophysical properties of the tumor microenvironment, such as tissue stiffness and fibroblast-mediated remodeling of the collagen extracellular matrix, can alter cell motility. For example, the loss of an important tumor suppressor, PTEN, in stromal fibroblasts leads to increased collagen deposition and remodeling, a stiffer microenvironment, and increased tumor growth (Trimboli, 2009). However, it is not known if "knocking down" MYOF expression (MYOF-KD) can modulate cell motility under different biophysical conditions. In this study, we first cultured spheroids of lentiviral control (LVC) and MYOF-KD tumor epithelial cells on polyacrylamide gels of varying stiffness and quantified migration over 24 hours. LVC cells cultured on stiffer gels exhibited greater migration while the migration of MYOF-KD cells was relatively low on all gels. In a second study, wild-type mouse mammary fibroblasts (WT-F) or fibroblasts with a mutation that results in the loss of PTEN (PTEN-/-) were seeded into a 2 mg/mL collagen gel and allowed to remodel the collagen matrix over 24 hours. Fluorescently labeled LVC and MYOF-KD epithelial cells were then seeded on top of the remodeled gels and invasion through the gel was quantified. PTEN-/- fibroblasts induced significantly more invasion for both LVC and MYOF-KD cells, and MYOF-KD cells invaded less than LVC cells under all conditions. In order to determine if this change in invasion was caused by a soluble factor, a conditioned media invasion assay was conducted with LVC and MYOF-KD cells cultured in media that had been

conditioned by WT-F or PTEN-/- fibroblasts. PTEN-/- conditioned media drove significantly more invasion than the media control in both the LVC and MYOF-KD cells, but the difference in invasion between the LVC and MYOF-KD cells was not significant. Additionally, the WT-F conditioned media resulted in a significant increase in invasion from the media control but only for the LVC cells. This indicates the possible presence of soluble factors secreted by PTEN-/- fibroblasts that affect both LVC and MYOF-KD cells. These results support that knocking down MYOF leads to decreased migration and invasion under different biophysical conditions. Future studies will seek to determine the specific mechanism by which knocking down MYOF alters cancer cell motility.

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1. Background

1.1 Introduction

The five year survival rate of localized breast cancer is 98.6%. However, this five year survival rate significantly decreases to 25.9% if the cancer has metastasized (Morris, 2015). Metastasis occurs when cancerous cells migrate away from the original tumor to other parts of the body. Many studies are being conducted to target cancer metastasis and develop treatments to inhibit cancer cell migration. The majority of these studies focus on the genetic predispositions or mutations that lead to cancer development. However, there are many other factors in the tumor microenvironment (TME) that facilitate cancer proliferation and invasion including changes in the extracellular matrix (ECM) as well as other chemical and mechanical factors. As cancer progresses, tumor epithelial cells and their microenvironment both change in ways that are conducive to tumor growth and invasion. Animal models usually only allow for variation and testing of genetic factors in tumor epithelial cells, and therefore little is known about how changes in the TME influence metastasis. In order to develop more effective treatments, a better understanding of the causes and mechanisms by which this migration occurs is necessary. This knowledge will provide greater insight for the development of potential treatments that can target and prevent tumor metastasis as well as aid in the creation of in vitro models that more accurately mimic in vivo conditions.

1.2 Myoferlin

Previous studies have shown that breast cancer cells exhibiting metastatic behavior often overexpress myoferlin (MYOF), a protein active in cell membrane functions such as repair and endocytosis. In a study of human mammary carcinomas, MYOF was overexpressed at various levels in all samples (Leung, 2013). Additionally, in a comparison between five cancerous

mammary cell lines (Fig 1-1), MYOF was shown to be expressed 2.44-fold more in highly invasive cell lines (Li, 2012).

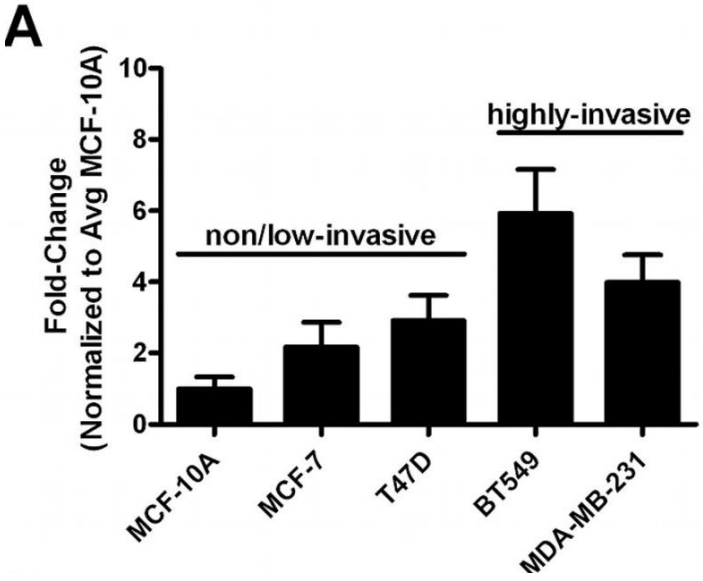

Figure 1-1 Myoferlin Overexpression: MYOF is overexpressed in all cancerous mammary cell lines tested, especially those that are highly invasive *(Li, 2012)*.

These studies indicate the need to further investigate MYOF's role in cancer progression and spread. One study hypothesized that because MYOF is highly involved in the regulation of epidermal growth factor receptor (EGFR), and dysfunctional EGFR has been linked to cancer cell proliferation, invasion, and migration, overexpression of MYOF could be conducive to these negative effects (Turtoi, 2013). Additionally, when MYOF is depleted in invasive breast cancer cell lines, the cells undergo metabolic stress making them more susceptible to drug targeting (Blomme, 2016). Similarly, Dr. Ghadiali's lab has depleted MYOF in a triple negative breast cancer cell line, MDA-MB-231, that indicated overexpression in MYOF as shown in Fig 1-1, by knocking down MYOF using a lentivirus. In an in vivo mouse model (Fig 1-2), this knockdown (KD) of MYOF in MDA-MB-231 breast cancer cells resulted in smaller tumors with less rough edges (Volakis, 2014).

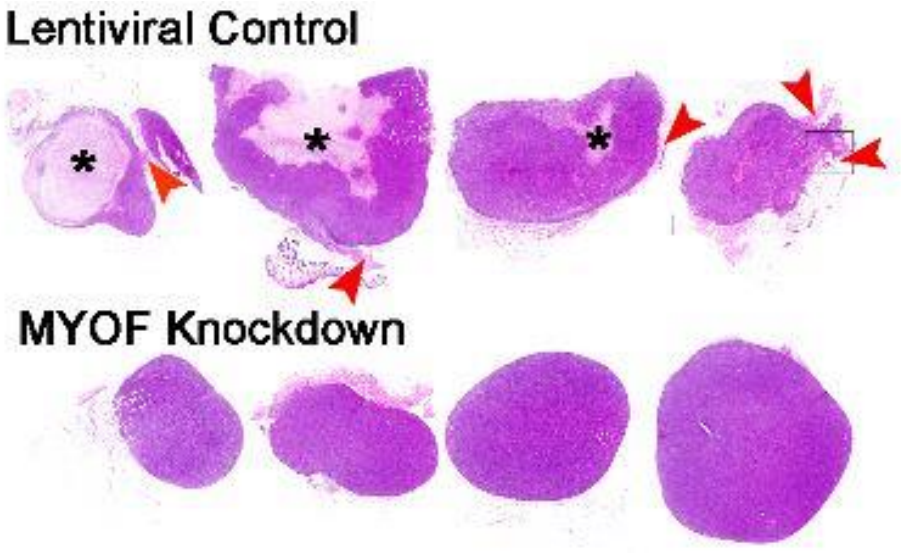

Figure 1-2 MYOF-KD In Vivo: H&E staining of tumor invasion (red arrows) and necrosis (black asterisks) indicate that KD of MYOF reduces such behavior *(Volakis, 2014).*

Dr. Ghadiali and his team have also conducted studies with the MDA-MB-231s in which the KD of MYOF reduced cell motility. However, most previous studies, especially those involving MYOF-KD like those shown in Figure 1-3, were only conducted on very stiff tissue culture plastic (TCP), which is not a good representation of the soft tissue conditions in vivo. TCP stiffness is on the magnitude of GPa; however, in a study conducted on mouse mammary tissue, normal breast tissue was found to be ~0.2 kPa while tumor tissue was measured to be ~2 kPa (Levental, 2009). Similarly on a study of human tissues, normal mammary tissue was measured to be .167 kPa and the average tumor to be 3-5 kPa as displayed in Table 1-2 (Paszek, 2005). The mechanism which allows the knockdown of MYOF to reduce cell motility is not yet completely understood and further testing is necessary prior to the development of methods with potential clinical significance.

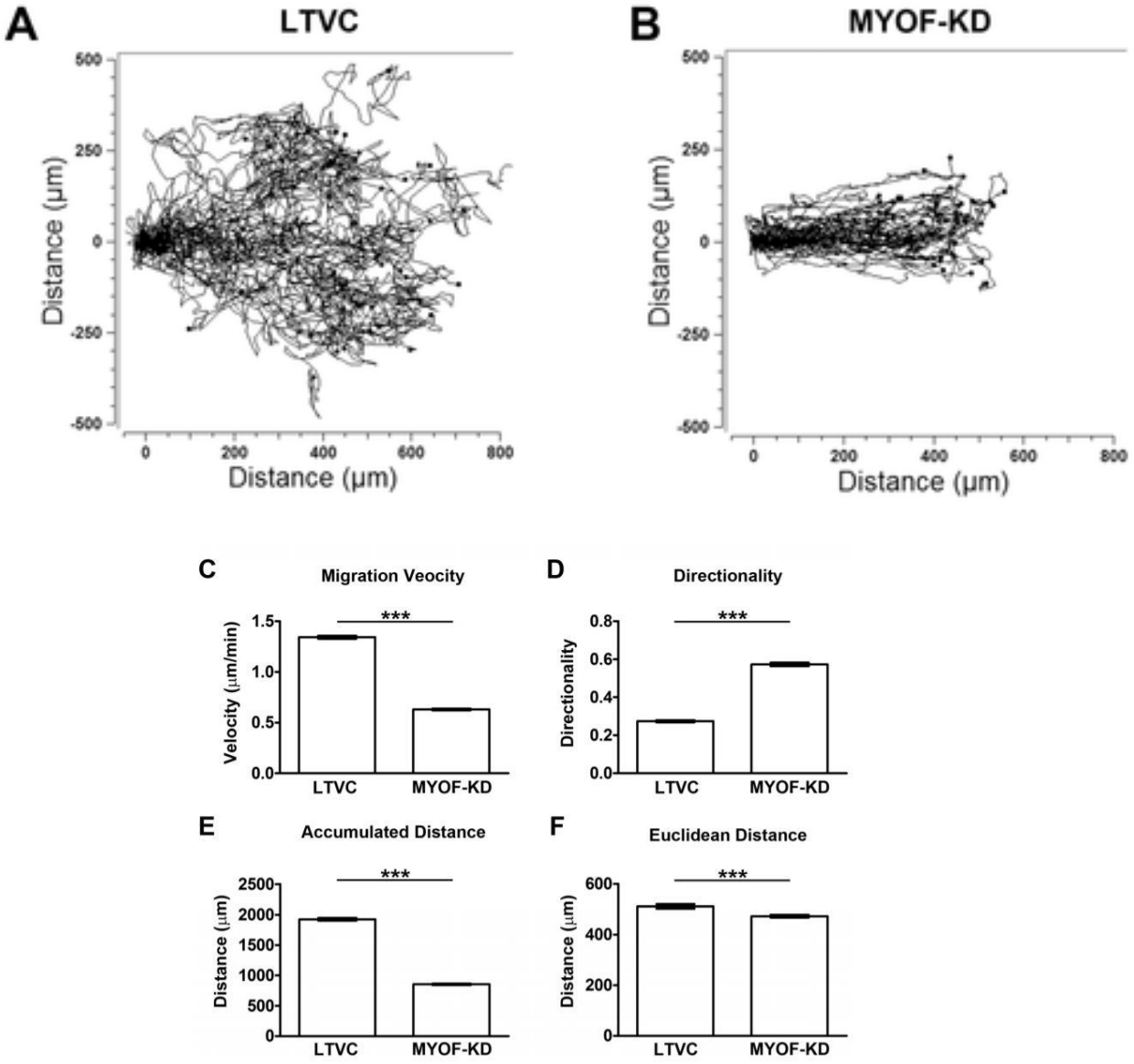

Figure 1-3 TCP Migration: Distance migrated by LVC (A) and MYOF-KD (B) MDA-MB-231 cells on TCP. C-F show quantified migration velocity, directionality, accumulated distance, and Euclidian distance respectively *(Volakis, 2014).*

Table 1-2 Elastic Moduli of Materials*:* The measured elastic moduli of materials and tissues relevant to this study *(Paszek, 2005)*.

Tissue or Material	Elastic Modulus (Pa)
Normal Mammary Gland	167 ± 31
Average Tumor (Ras, Myc, Her2/Neu)	4049 ± 938 **
Stroma Attached to Tumor (Ras, Her2/Neu)	918 ± 269 **
Reconstituted Basement Membrane	175 ± 37
Collagen (2.0 mg/ml)	328 ± 87
Collagen (4.0 mg/ml)	1589 ± 380
Plastic (polystyrene) (Callister et al., 2000)	2.78×10^9
Glass (soda-lime) (Callister et al., 2000)	69×10^9

** Mean ± SEM, $p<0.01$

As shown in Figure 1-3, previous studies with knocking down MYOF resulted in less accumulated distance traveled compared to LVC cells. Additionally, MYOF-KD cells had decreased migration velocity but increased directionality, indicating that they migrate more slowly in a less randomized manner than LVC cells (Volakis, 2014). This behavior is more typical of epithelial cells than transformed tumor cells and could indicate that the knockdown of MYOF induces a change in cell phenotype.

1.3 Epithelial to Mesenchymal Transition

One hypothesis is that the knockdown of MYOF induces cells into a mesenchymal to epithelial transition (MET). The opposite process, epithelial to mesenchymal transition (EMT), occurs when several factors including changes in polarity and loss of cell-cell adhesion result in once epithelial cells adopting a mesenchymal like phenotype. EMT occurs naturally in the body when epithelial cells that normally interact with their basement membrane adopt a more mesenchymal phenotype, and is a necessary component of embryogenesis and wound healing (Kalluri, 2009). However, when this behavior occurs during cancer progression it allows cells to become more migratory and can facilitate metastasis. In addition, cancer cells exhibit several

biomechanical properties during EMT including increases in cytoplasmic stiffness and contractile forces. These morphological and biophysical changes allow cancer cells to migrate away from each other and invade into other tissues (Lamouille, 2014). There are many signals both endocrine and mechanical (Fig 1-4) that are known to induce EMT (Yeung, 2016). However, the effect of knocking down MYOF on EMT in invasive breast cancer cells is not yet fully understood.

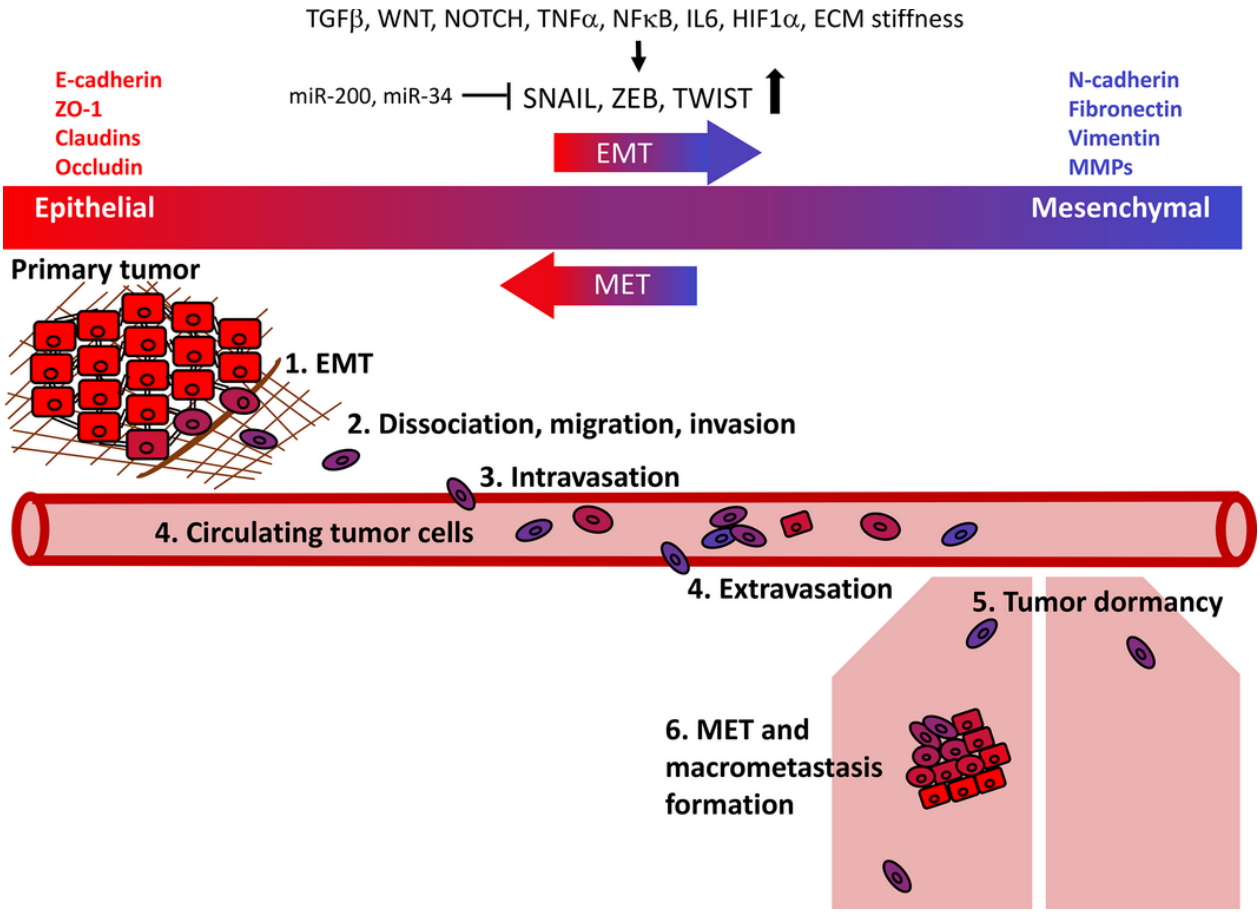

Figure 1-4 Epithelial to Mesenchymal Transition: A schematic on known EMT inducers and markers. When cells undergo EMT they are more likely to become metastatic. *(Yeung, 2016)*

Known regulators of EMT include not only the ECM stiffness of the mechanical environment, but transcriptional factors and other small molecules as well. In a study of patient derived metastatic breast cancer cells, SNAIL1, TWIST1, and SLUG overexpression resulted in poor prognosis for patients (Imani, 2016) . Additionally, the proteins E-Cadherin (E-cad) and vimentin have been demonstrated to be markers of EMT in invasive breast cancer cell lines For

example, western blot analysis (Fig 1-5) of MDA-MB-231 cells grown on TCP have shown changes in these common EMT markers when MYOF is knocked down (Volakis, 2014). Vimentin is downregulated and E-cadherin (E-cad) is upregulated, which both indicate the MYOF-KD cells no longer exhibit the mesenchymal phenotype of the control cells. Vimentin is often highly expressed in mesenchymal cells and has been shown to contribute to the reorganization of the cytoskeleton and stability of mesenchymal cells that have undergone EMT (Liu, 2015). In contrast, the cell adhesion molecule E-cad is commonly expressed in cells of epithelial phenotype and its loss likely contributes to increased invasive capacity of mesenchymal cells (Larue, 2005).

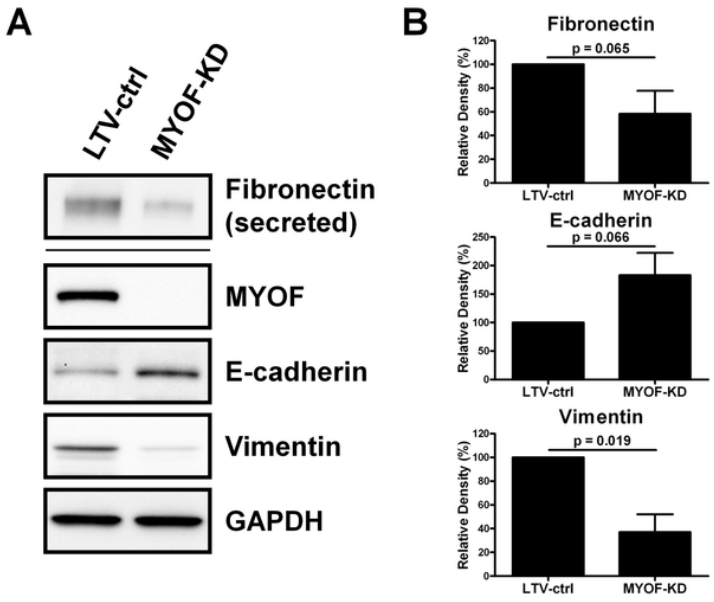

Figure 1-5 Western Blot on TCP: Western blot of EMT markers in LVC and MYOF-KD cells *(Volakis, 2014).*

In addition to these molecular changes in phenotype, EMT has been shown to induce other changes as well. As mentioned, the loss of E-cad contributes to reduced cell adhesion to each other and the ECM, but previous studies have found that when MYOF is depleted in breast cancer cells, they appear to regain their adhesion forces reducing their motility (Blackstone, 2015). EMT can also contribute to changes in morphology. Cells that have undergone EMT often lose their

epithelial shape and become elongated, resembling spindle shaped fibroblasts (Marck, 2000). However when MYOF is knocked down in MDA-MB-231 cells (Fig 1-6), they regain their epithelial morphology (Li, 2012).

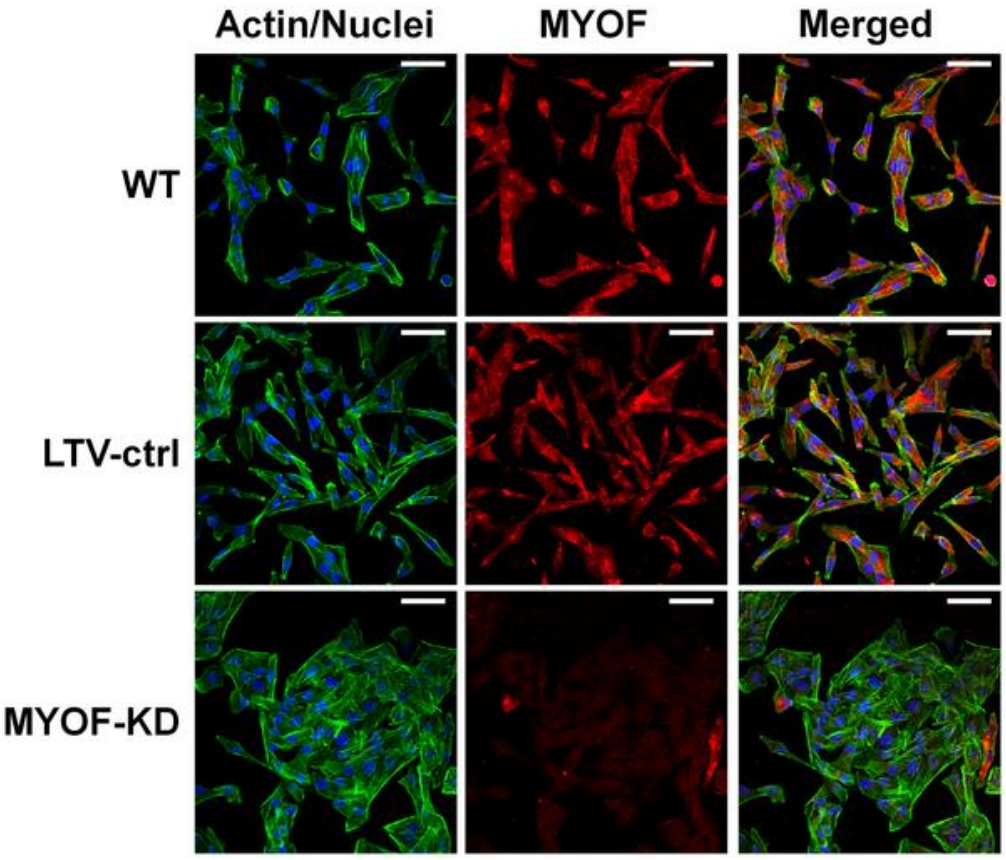

Figure 1-6 Morphology Changes in MYOF-KD Cells: Immunofluorescence images that show the morphology change MYOF-KD induces. Cells become less elongated and exhibit a more epithelial phenotype *(Li, 2012)*.

Clearly there is great indication of the ability of MYOF-KD to reduce the invasive behavior of MDA-MB-231 cells and revert them back to an epithelial phenotype. However, as mentioned before, these studies were all conducted on TCP which is an environment that is much stiffer than in vivo mammary conditions. Stiffness plays a key role in mammary tumor formation and higher in vivo tumor stiffness has been correlated to an increase in metastasis (Fenner, 2014). It is therefore necessary to investigate if MYOF-KD cells still exhibit reduced EMT behavior such as migration when cultured on an environment more similar to in vivo conditions through the use of

polyacrylamide (PA) gels. It is hypothesized that **substrate stiffness will induce changes in the migration of LVC and MYOF-KD cells**

1.4 Cancer Associated Fibroblasts

Simply varying stiffness with PA gels fails to incorporate the mechanisms behind what causes these changes in stiffness in vivo, as well as stiffness changes that continue to occur during tumor formation and invasion. A more dynamic model would be advantageous in analyzing the true efficacy of MYOF depletion as a tool to inhibit metastasis.

Stromal fibroblasts, cells that are located in the tumor microenvironment (TME), are responsible for secreting and interacting with ECM proteins such as collagen, and become activated during cancer formation (Erdogan, 2017). If mutated in some way, these fibroblasts can induce radial alignment of collagen fibers which generates pathways that are conducive for cell migration and invasion. In addition, this rise in collagen deposition has been shown to correlate with an increase in stiffness of the TME (Schedin, 2011). It has been shown that mammary stromal fibroblasts with a mutation that results in the loss of phosphate tensin homolog (PTEN) resulted in many oncogenic behaviors (Trimboli, 2009). In addition to increased tumor formation in vivo with mouse models (Fig 1-7), the inactivation of PTEN (PTEN-/-) resulted in heavy ECM remodeling and increased angiogenesis which are all factors conducive to enhanced migration. PTEN deletion in mouse mammary cells has also been shown to increase collagen deposition which is conducive to cell migration (Wallace, 2011).

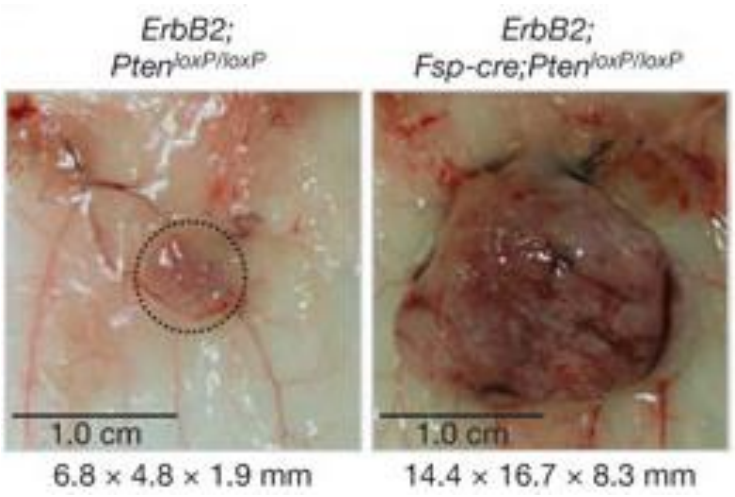

Figure 1-7 In Vivo PTEN Inactivation: Mouse models showed increased tumor formation when PTEN had been inactivated *(Turtoi, 2013)*.

Using the stromal fibroblasts PTEN-/- and WT-F cells in conjunction with the MYOF-KD and LVC epithelial cells will produce an in vitro environment that better mimics the actual TME than studies previously conducted. It is hypothesized that **PTEN-/- fibroblasts will increase the invasive capacity of LVC and MYOF-KD cells**

The ideas behind more advanced in vitro models like the one proposed could be applied to other types of cancer as well, especially those of epithelial cell origin. An in vitro model that more accurately captures in vivo conditions would allow for faster discovery of the mechanisms by which cancer develops and spreads. This would provide greater insight for the development of methods to counteract the proliferation and migration of cancer cells. Improved models could also hasten drug development and testing by providing more precise data on the efficacy of a proposed cancer drug. For instance, if the hypothesis that knocking down MYOF reduces invasion is supported by the model, it would indicate that MYOF-KD would be able to reduce 3D cell motility in vivo and should continue to be pursued as a potential therapy to inhibit metastasis, possibly

through small molecule inhibitors of MYOF that could be incorporated into a treatment regime for patients with metastatic breast cancer.

2. Materials and Methods

2.1 Cell Culture

Highly invasive, triple negative MDA-MB-231 cells derived from a pleural effusion were utilized throughout this study. The parental wild-type (WT) line, MDA-MB-231^{WT}, was used to make MYOF knockdown, MDA-MB-231^{KD,} cells using a lentiviral based delivery with interfering RNAs directed against human MYOF and a stable lentiviral control (LVC), MDA-MB-231^{LVC}, was created with a non-targeting shRNA (Li, 2012). All MDA-MB-231 cells lines were a kind gift from the lab of Dr. Kniss. Mouse mammary PTEN-null (PTEN-/-) and WT-F stromal fibroblasts gifted from Dr. Michael Ostrowski's lab were also kept in culture and used for portions of this study. All cell lines were kept in incubation (37°C, 5% CO_2, 95% humidity) on tissue culture polystyrene (TCP) with high-glucose (4.5 g/l) Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 5% Antibiotic/Antimycotic (Anti/Anti).

2.2 Polyacrylamide Gel Molding

Polyacrylamide (PA) gels of varying stiffness were made according to a protocol published previously (Tse, 2010). Table 2-1 below outlines the recipe used to make the 0.5, 2, and 20kPa gels. In addition to the values of acrylamide (Bio-Rad) and bis-acrylamide (Bio-Rad) in the table, 10 µl of ammonium persulfate (Sigma-Aldrich) and 1 µl of Tetramethylethylenediamine (Bio-Rad) were added to each gel mixture to aid with polymerization.

Table 2-1 Polyacrylamide Gel Recipe: Procedure to make 1 mL of each stiffness gel (Tse, 2010).

Acrylamide %	Bis-acrylamide %	Acrylamide from 40% stock solution (µl)	Bis-acrylamide from 2% stock solution (µl)	Water (µl)	E ± St. Dev. (kPa)
3	0.06	75	30	895	0.48 ± 0.16
4	0.1	100	50	850	2.01 ± 0.75
8	0.264	200	132	668	19.66 ±1.19

96.8 µL of gel mixture was placed between a non-treated glass slip and a 22x22 mm treated glass coverslip to create a polyacrylamide (PA) gel of 0.2 mm in thickness. These coverslips were treated by flaming to sterilize, adding 200 µl of 0.1 N NaOH until evaporation, 100 µl of 3-aminopropyltrimethoxylane (Sigma-Aldrich) for 5 min, and a solution of 0.5% Glutaraldehyde for 30 min. Gels were polymerized for ~1 hour after which the non-treated glass slip was removed and the gels were stored in deionized (DI) water at +4°C. One day prior to MDA-MB-231 cell seeding, the PA gels were coated with 200 µL of 200 µg/ml bovine collagen (Advanced BioMatrix) in 2 mM HCl for one hour after treating with 200 µl of 0.5 mg/ml of Sulfo Sanpah solution (Thermo Scientific) in HEPES under a UV lamp for 5 mins twice. The gels were sterilized prior to cell seeding by washing with ethanol and sterile phosphate buffer solution (PBS).

2.3 24-Hour Migration Experiments

LVC and MYOF-KD MDA-MB-231 cell lines were made into cell clusters using the hanging drop method in which 10 µL droplets of ~1000 cells in DMEM are placed onto a polydimethylsiloxane (PDMS) gel and inverted for 24 hours in incubation (37°C, 5% CO_2, 95% humidity). Adhesion forces keep the droplet on the PDMS and gravity pulls the cells to the tip of the droplet forcing them to adhere to each other and form a cluster. These clusters were then seeded onto the PA gels of 0.5, 2, and 20 kPa stiffness to allow for outward migration (Fig. 2-1).

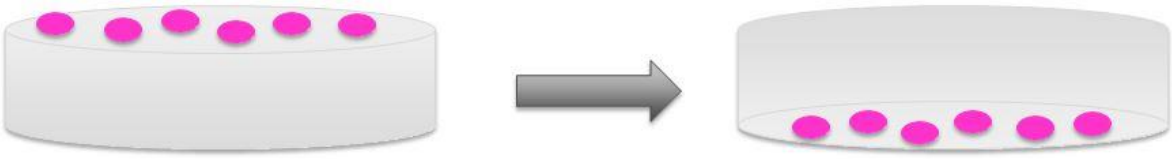

Figure 2-1 Hanging Drop Schematic: The adhesion forces of the media allowed the droplets to stick the PDMS and gravity pulls the cells to the base of the droplet forcing them to adhere to each other.

The gels were placed in a live cell imaging system (37°C, 5% CO_2, 95% humidity) and images of their migration were captured every ten minutes for 24 hours with an Olympus Ix81 microscope at 10X objective. Images every 4 hours were analyzed in ImageJ to determine the migration patterns of the MDA-MB-231 cells. Each cell cluster was normalized by determining the centroid point (x_c, y_c) of the cluster at time 0. Coordinate points (x_t, y_t) were collected from all migrating cells (n) at each four hour time point and average migrating distance was calculated using Equation 1 below. Distance was not tracked cell by cell because as cells migrated outward from the cluster, more became visible and were included in the distance calculation. The median distance migrated of each cluster was determined and used for further statistical analysis.

$$Distance = \sqrt{(x_t - x_c)^2 + (y_t - y_c)^2} \quad (1)$$

2.4 Western Blots

LVC and MYOF-KD MDA-MB-231 cells lines were seeded at a density of $2x10^5$ cells/gel onto 0.5, 2, and 20 kPa gels in a monolayer and kept in incubation (37°C, 5% CO_2, saturated humidity) for 48 hours. The cells were then rinsed with PBS and lysed for 30 mins in cold lysis buffer made up of 500 µL RIPA (radioimmunoprecipitation assay) buffer, 75 µL mini protease cocktail (Roche), 5 µL phosphatase inhibitor cocktail 2 (Life Science) , and 10 µL phenylmethane sulfonyl fluoride. The cell lysate was centrifuged for 15 min at 13,000 rpm at 4°C. The protein concentration of each supernatant was determined using a bicinchoninic acid (BCA) Protein Assay Kit (Thermo Scientific) with bovine serum albumin as standards in order to load 30 µg per lane into a Mini-PROTEAN® TGX Precast Gel (Bio-Rad). The protein was transferred to a 0.45 µm nitrocellulose membrane (Bio-Rad) and probed with primary antibodies Actin (Cell Signaling), Myoferlin (Abcam), E-Cadherin (BD Biosciences), and Vimentin (Cell Signaling) in tris-buffered saline containing 0.1% Tween-20 and 5% non-fat dry milk for 24 hours. After washing,

membranes were treated with either anti-mouse (Actin, MYOF, and E-Cad) or anti-rabbit (Vimentin) secondary antibodies (Bio-Rad) in tris-buffered saline containing 0.1% Tween-20 and 5% non-fat dry milk for 1 hr. Bands were revealed with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) and imaged with VersaDoc Imaging System and analyzed with QuantityOne analysis software (Bio-Rad, Hercules, CA).

2.5 Collagen Invasion Assay

The invasion assay was prepared using a transwell insert (Corning Life Sciences) with two layers of a 2 mg/mL bovine collagen (Advanced Biomatrix) gel. Layer one is prepared with 1000 µL of 6 mg/mL collagen, 300 µL PBS, 10µL 1M NaOH, and 1690 µL Sterile Water and 300 µL of the solution is placed into each of the transwell inserts and polymerized in incubation for 20 minutes. The second layer was prepared with 1440 µL of 6 mg/ml collagen, 360 µL of PBS, 14.4 µL of NaOH, 1785.6 µL of sterile water, and 300 µL of either DMEM or $6x10^4$ cells PTEN-/- or WT-F mouse mammary stromal fibroblasts was placed into each of the Boyden chambers. The transwell inserts were then kept in incubation (37°C, 5% CO_2, 95% humidity) for 24 hours to allow the fibroblasts to remodel the matrix.

LVC and MYOF-KD MDA-MB-231 cells were treated with a CFDA (Life Technologies) for 30 mins after which the cells were passaged and $2.5x10^5$ cells were placed on top of the insert and allowed to invade for 24 hours. Fluorescent images of the bottom of the insert were taken at 10X with Olympus Ix81 microscope and number of invading cells were quantified.

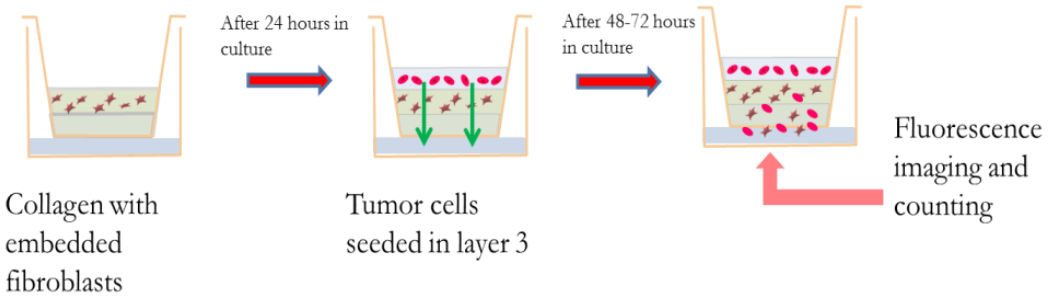

Figure 2-2 Collagen Invasion Assay Schematic: Schematic of fibroblasts and MDA-MB-231 cells in a transwell insert.

2.6 Conditioned Media Invasion Assay

Collagen invasion assays were made with the same procedure as above, but instead of having two different layers, two layers of the same 2 mg/mL collagen solution were used. This solution was made with 1000 µL of 6 mg/mL collagen, 300 µL PBS, 10 µL 1 M NaOH, and 1690 µL sterile water and 300 µL of the solution is placed into each of the transwell inserts and polymerized in incubation for 20 minutes. The second layer was then put on and allowed to polymerize in incubation for an additional 20 minutes. Although each layer of collagen gel solution was the same, two layers were used to keep experimental protocol consistent with the invasion assay with fibroblast gel remodeling discussed above. $2.5x10^5$ cells of either CFDA treated LVC or MYOF-KD MDA-MB-231 cells were placed on the gel and allowed to invade for 24 hours in incubation. However, instead of culturing these cells in the typical DMEM used in incubation (10% FBS and 1% Anti/Anti) conditioned media from WT-F and PTEN-/- mouse mammary fibroblasts was used. This media was made by culturing $1.2x10^5$ cells WT-F and PTEN-/- fibroblasts in 6 mL serum free (no FBS) DMEM on TCP for 48 hours. After 24 hrs of invasion, fluorescent images of the bottom of the insert were taken at 10X with Olympus Ix81microscope and number of invading cells were quantified.

2.7 Statistical Analysis

All statistical analysis was conducted in Sigma Plot v.13 (Systat). A Two-Way ANOVA followed by a Holm-Sidak Comparison Method was conducted for all migration analysis with $\alpha=0.05$. First, the median distance traveled by a cluster's cells was found. Then the medians were analyzed together and the mean of these values ± standard deviation are reported in the graphs in Chapter 3.1 for n=3 experiments. For the collagen invasion and conditioned media assays, a Two-Way ANOVA was conducted with a Holm-Sidak comparison method and $\alpha=0.05$. The mean number of invading cells ± standard deviation are reported in the graphs in Chapter 3.2 for n=3 experiments.

3. Results

3.1 The Effect of Substrate Stiffness on MDA-MB-231 Cell Migration

Although MYOF-KD has been shown to reduce EMT behaviors on TCP, this stiffness is not representative of in vivo body stiffness and PA gels of 0.5, 2, and 20 kPa stiffness were used to create an environment more similar to body conditions. Images of migrating cells were taken every ten minutes, however only images at time points 0, 4, 8, 12, 16, 20, and 24 hours were used during analysis. Below are representative images of each cluster type at these time points (Figs 3-1 through 3-6). In some instances it is difficult to simply visualize differences among cluster types because although all clusters were made to be ~1000 cells, many broke during the transport from the PDMS gel to the PA gels resulting in somewhat inconsistent cluster size. This inconsistency was addressed during quantification of distance migrated by calculating an original centroid point of each cluster and subtracting it from the migrated coordinate points used in distance calculations. The most obvious difference in the images is that as stiffness of the gel lowers, the overall migration of the cells is also reduced. The 0.5 kPa gel for instance visibly has the least migration for both LVC and MYOF-KD cells.

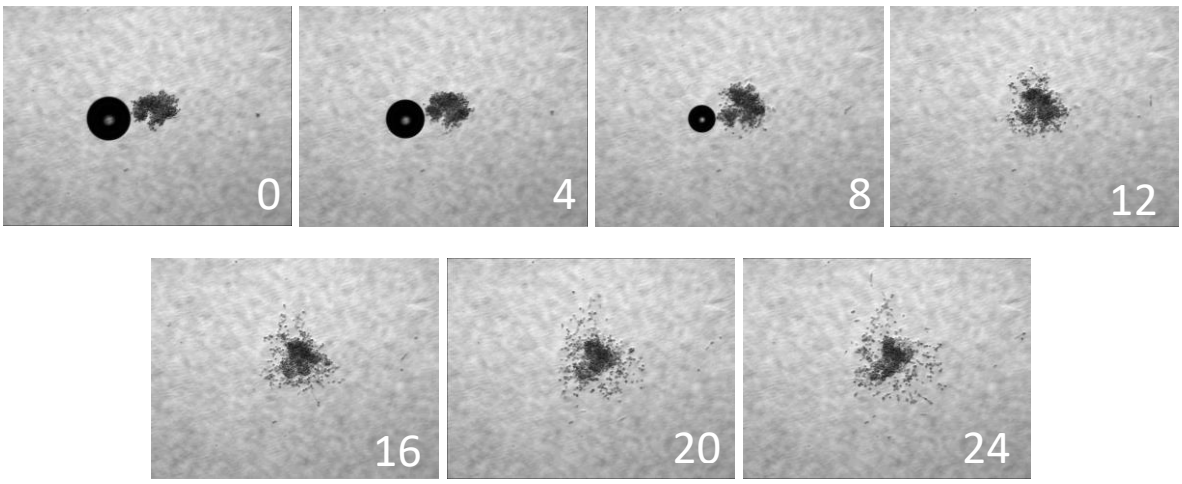

Figure 3-1 LVC 0.5 kPa Gel: Cell cluster at time points every 4 hours for 24 hours for LVC cells on 0.5 kPa gel.

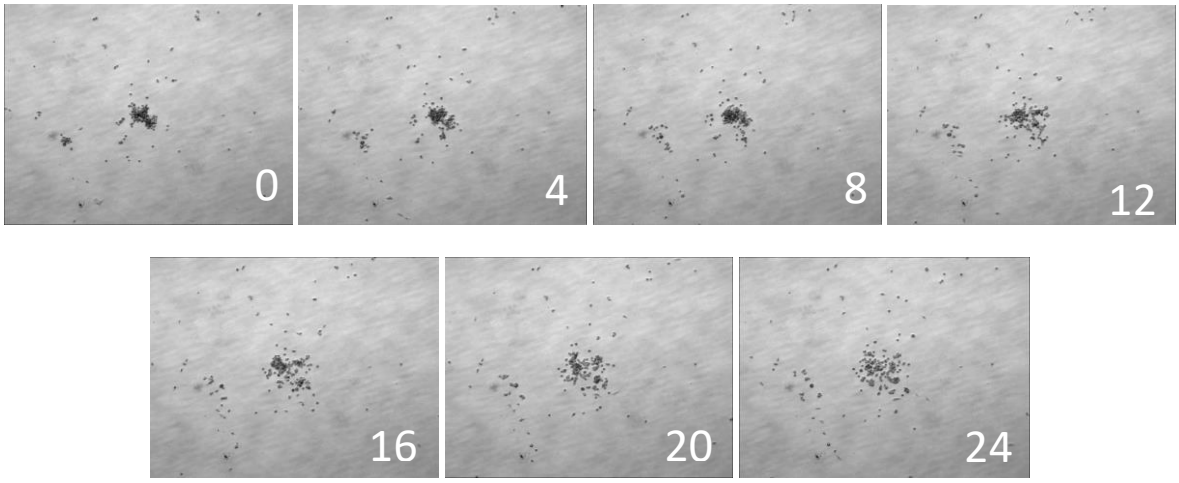

Figure 3-2 MYOF-KD 0.5 kPa Gel: Cell cluster at time points every 4 hours for 24 hours for MYOF-KD cells on 0.5 kPa gel.

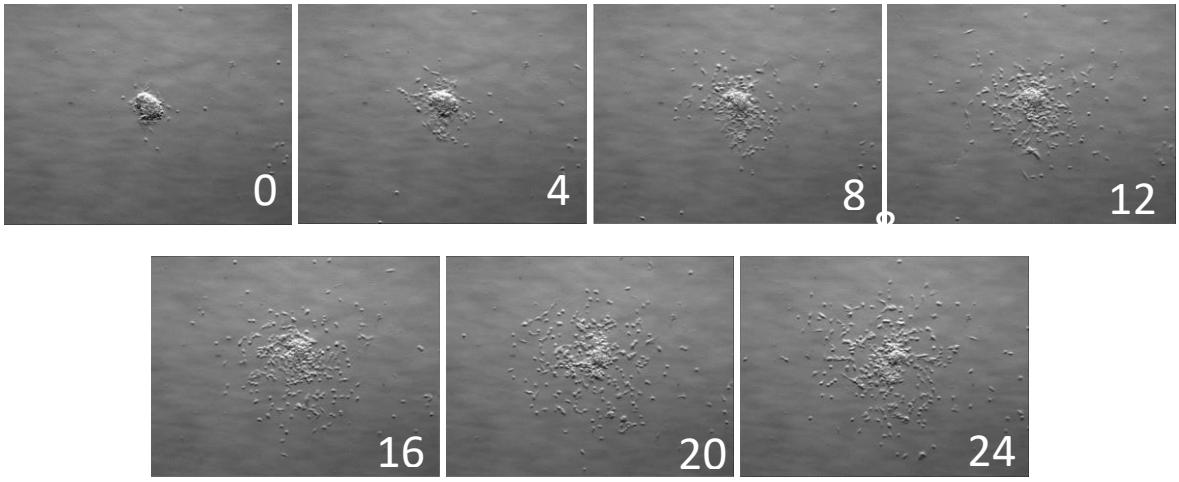

Figure 3-3 LVC 2 kPa Gel: Cell cluster at time points every 4 hours for 24 hours for LVC cells on 2 kPa gel.

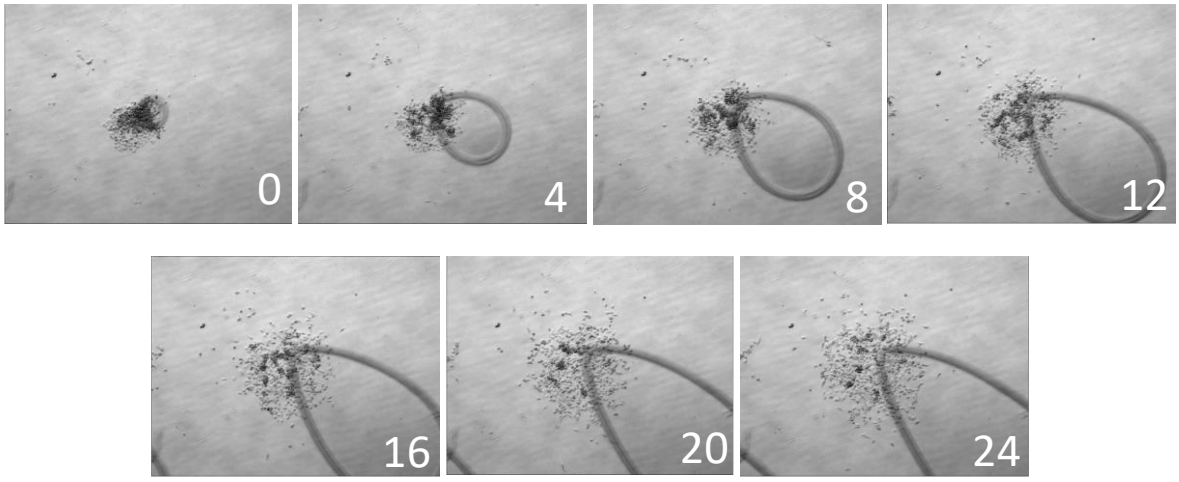

Figure 3-4 MYOF-KD 2 kPa Gel: Cell cluster at time points every 4 hours for 24 hours for MYOF-KD cells on 2 kPa gel.

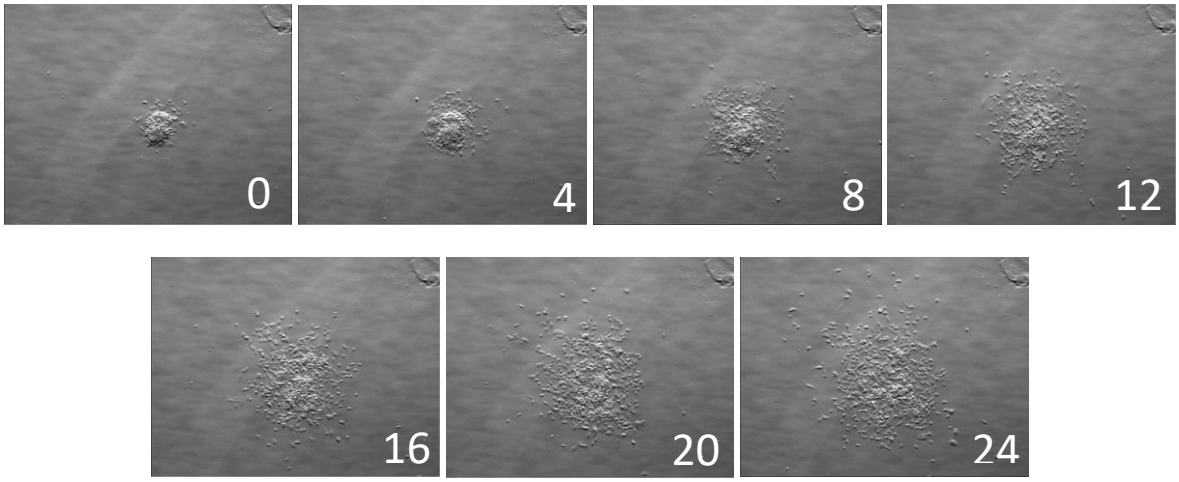

Figure 3-5 LVC 20 kPa Gel: Cell cluster at time points every 4 hours for 24 hours for LVC cells on 20 kPa gel.

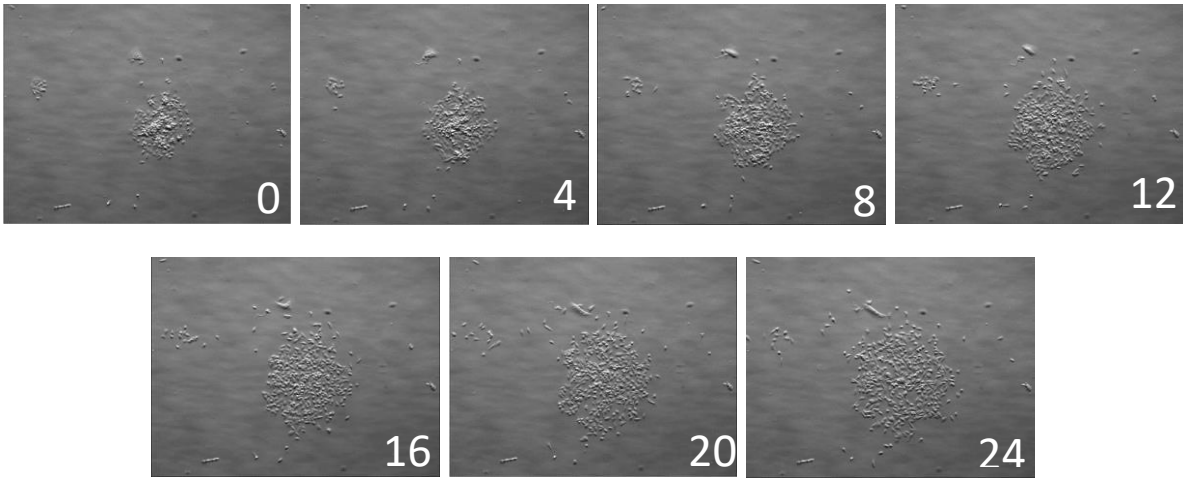

Figure 3-6 MYOF-KD 20 kPa Gel: Cell cluster at time points every 4 hours for 24 hours for MYOF-KD cells on 20 kPa gel.

In order to get a better understanding of the true migration of each cluster, the distance migrated for each cell was quantified. The median of these values for each cluster was determined and the average of these medians ± standard deviation is shown on the graphs (Figures 3-7 through 3-11). Figure 3-7 and 3-8 show the change in migration caused by each stiffness gel on LVC and MYOF-KD cells respectively. For LVC cells, as stiffness increases, migration increases as well. After 12 hours the distance migrated for LVC cells on 20 kPa gels is significantly greater than that of the LVC cell on 0.5 kPa gel. The difference in migration between the 2 kPa and 20 kPa gel is not nearly as drastic, but by 24 hours, the 20 kPa gel induced significantly greater migration on

LVC cells than the 2 kPa gel. For MYOF-KD cells there are no statistically significant differences in migration among the varied stiffness gels.

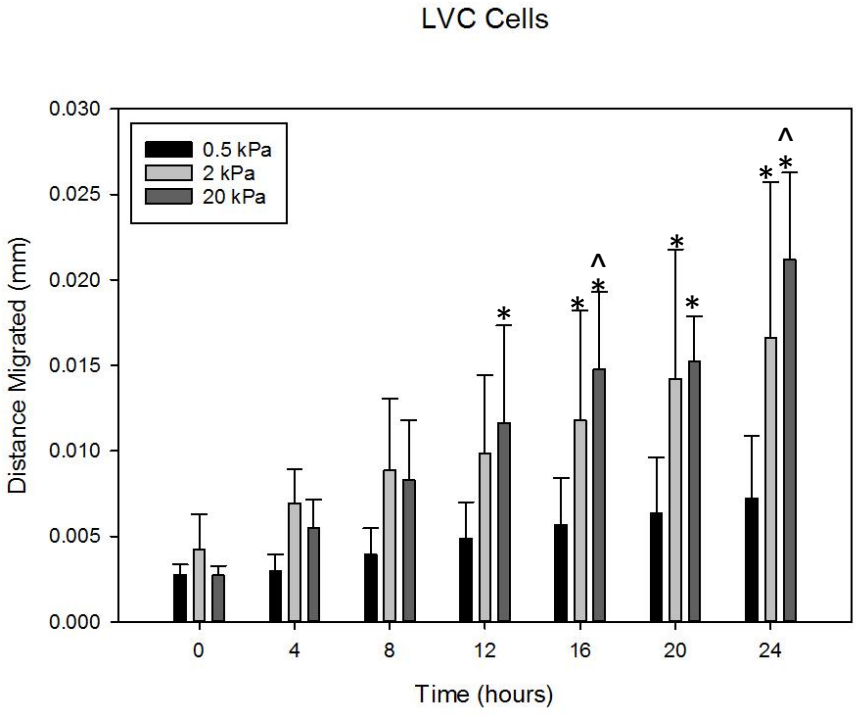

Figure 3-7 LVC Migration Data: Quantification of distance migrated of LVC cells on all stifness gels. * Indicates statistical significance (p=.05) in distance migrated between 0.5 kPa gel and either 2 or 20 kPa gel. ^ Indicates statistical significance in distance migrated between 2 and 20 kPa gel.

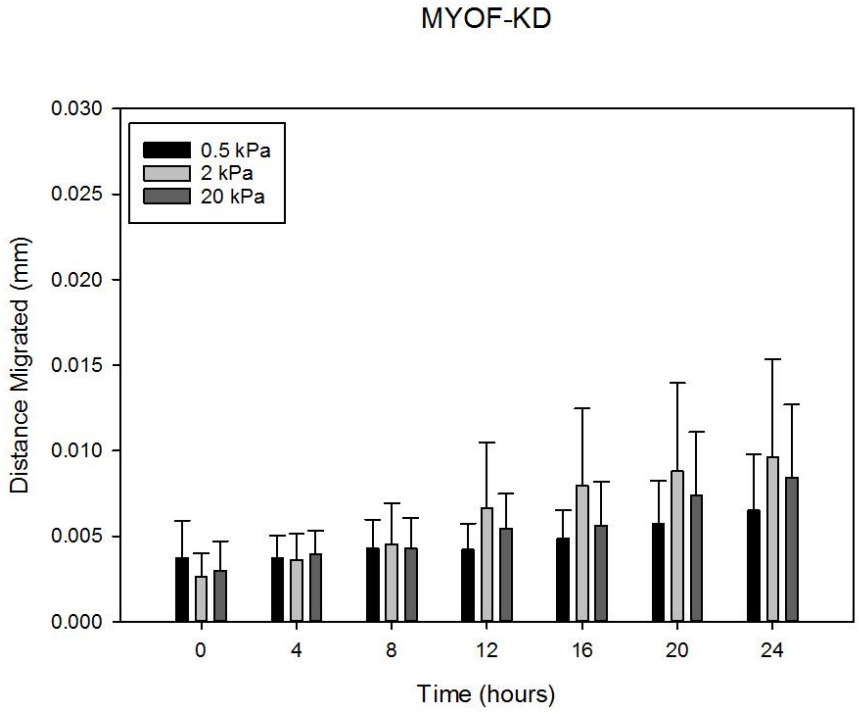

Figure 3-8 MYOF-KD Migration Data: Quantification of distance migrated of MYOF-KD cells on all stifness gels. There was no statistically significant difference in migration distance for all stiffness gels.

Figures 3-9, 3-10, and 3-11 show the differences between LVC and MYOF-KD cell migration on 0.5, 2, and 20 kPa stiffness gels respectively. There is significantly more migration by the LVC cells than the MYOF-KD cells by 24 hours on both the 2 kPa and 20 kPa gel. However, this is not the case for the 0.5kPa gel. Even at 24 hours, the distance migrated by the LVC and MYOF-KD cells is not significantly different. This indicates that as hypothesized, LVC cells are able to migrate faster on stiffer environments and that the knockdown of MYOF is effective in reducing migration regardless of stiffness.

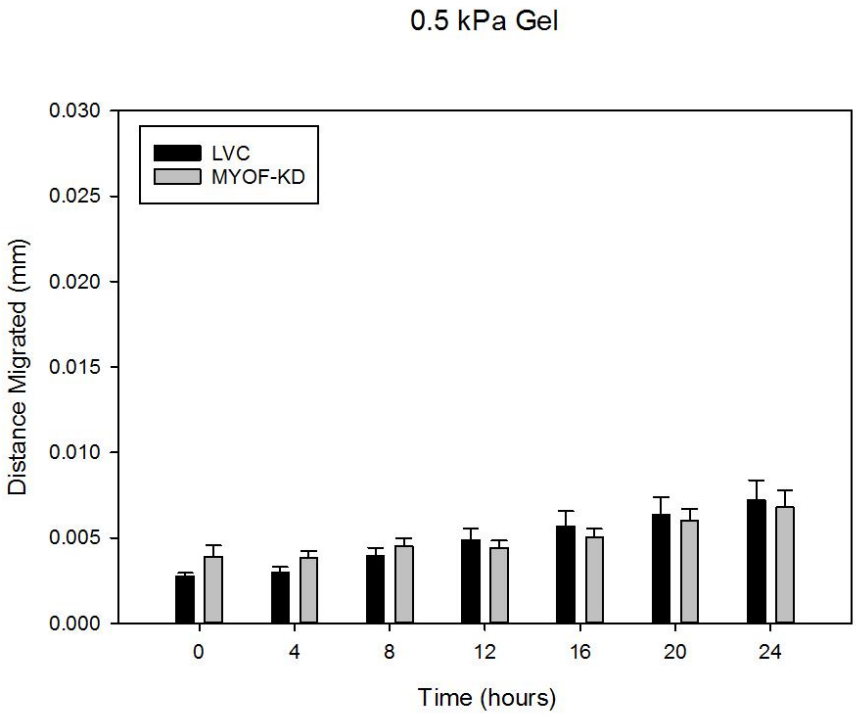

Figure 3-8 0.5 kPa Migration Data: Quantification of distance migrated of LVC and MYOF-KD cells on 0.5 kPa gels. There was no statistically significant difference in migration between LVC and MYO-KD cells on the 0.5 kPa gel.

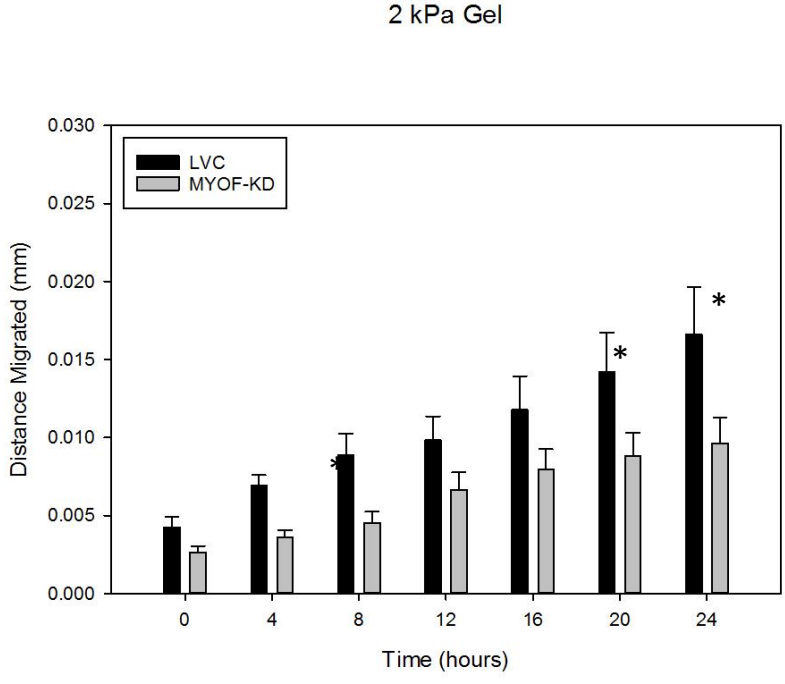

Figure 3-9 2 kPa Migration Data: Quantification of distance migrated of LVC and MYOF-KD cells on 2 kPa gels. * Indicates statistical significance (p=.05) in distance migrated between LVC and MYOF-KD cells on the 2 kPa gel.

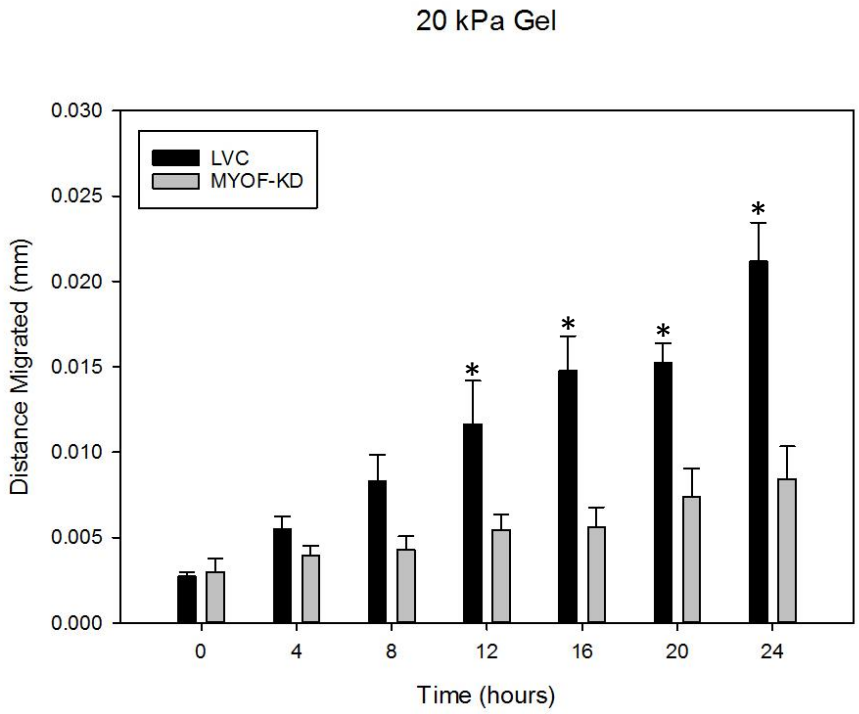

Figure 3-11 20 kPa Migration Data: Quantification of distance migrated of LVC and MYOF-KD cells on 20 kPa gels. * Indicates statistical significance (p=.05) in distance migrated between LVC and MYOF-KD cells on the 20 kPa gel.

In order to determine if gel stiffness could revert mesenchymal cells back to an epithelial phenotype, a western blot was conducted on the common EMT markers E-Cadherin (E-cad) and vimentin as well as MYOF and an Actin loading control. E-Cad has been shown to be up regulated in epithelial cells. Below are images of the bands (Fig 3-12). As expected, MYOF was only present in the LVC cells and not the MYOF-KD cells. In addition, E-Cad was present in MYOF-KD cells but not LVC cells.

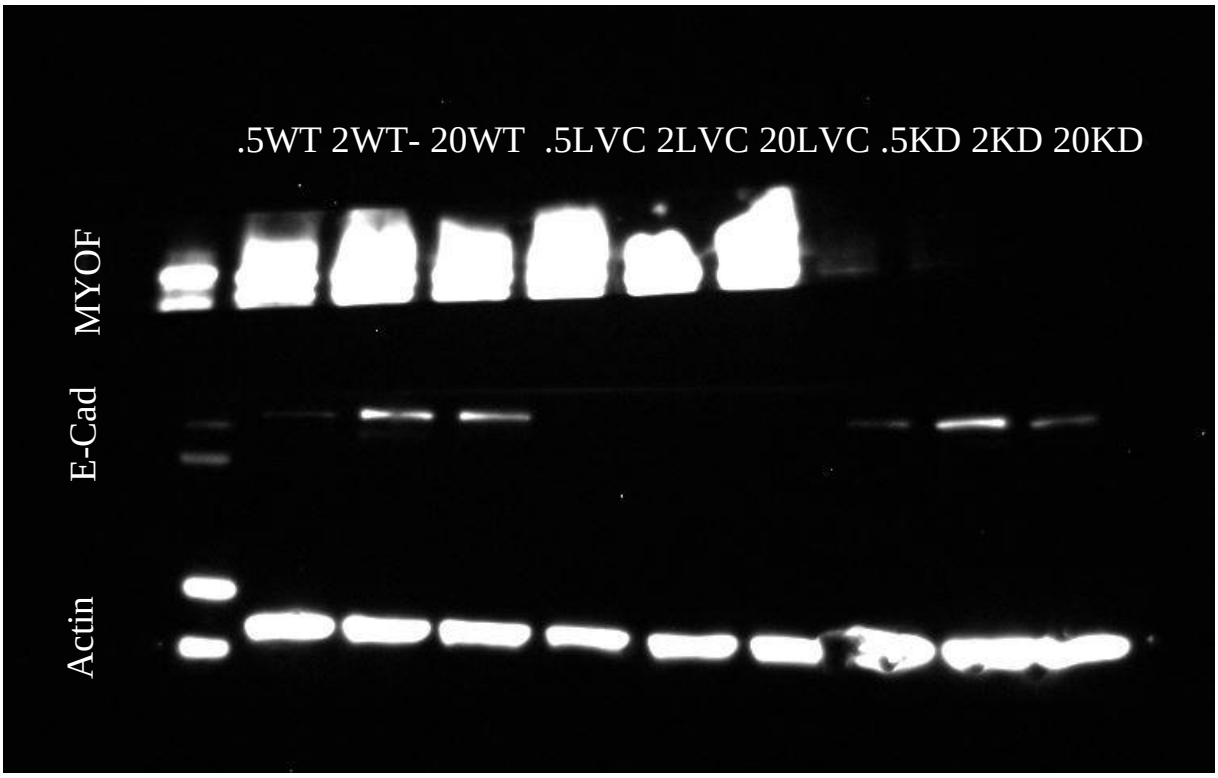

Figure 3-12 Western Blot: Western blot of MYOF, E-Cad and Actin concentration in WT-F, LVC, and MYOF-KD cells cultured on 0.5, 2, and 20 kPa gels.

Due to experimental complications, this is the only western blot completed at the moment. In the future, more samples will be tested and vimentin will be analyzed as well. No additional data analysis could be conducted because this is only n=1.

3.2 Effect of Stromal Fibroblasts on MDA-MB-231 Cell Invasion

Changes in stiffness is not the only change a tumor microenvironment undergoes during tumor development, fibroblast activity is increased as well. PTEN-/- stromal fibroblasts have been shown to increase tumorigenesis. A collagen invasion assay was conducted to determine the effect of PTEN-/-, a cancer associated fibroblast, on MDA-MB-231 LVC and MYOF-KD cells' invasion. Florescent images of invading cells are shown in Fig 3-13. It is clear from the images that the MYOF-KD cells invaded less than LVC MDA-MB-231 cells. However, it is difficult to determine simply from the figures any significant changes among different fibroblast types.

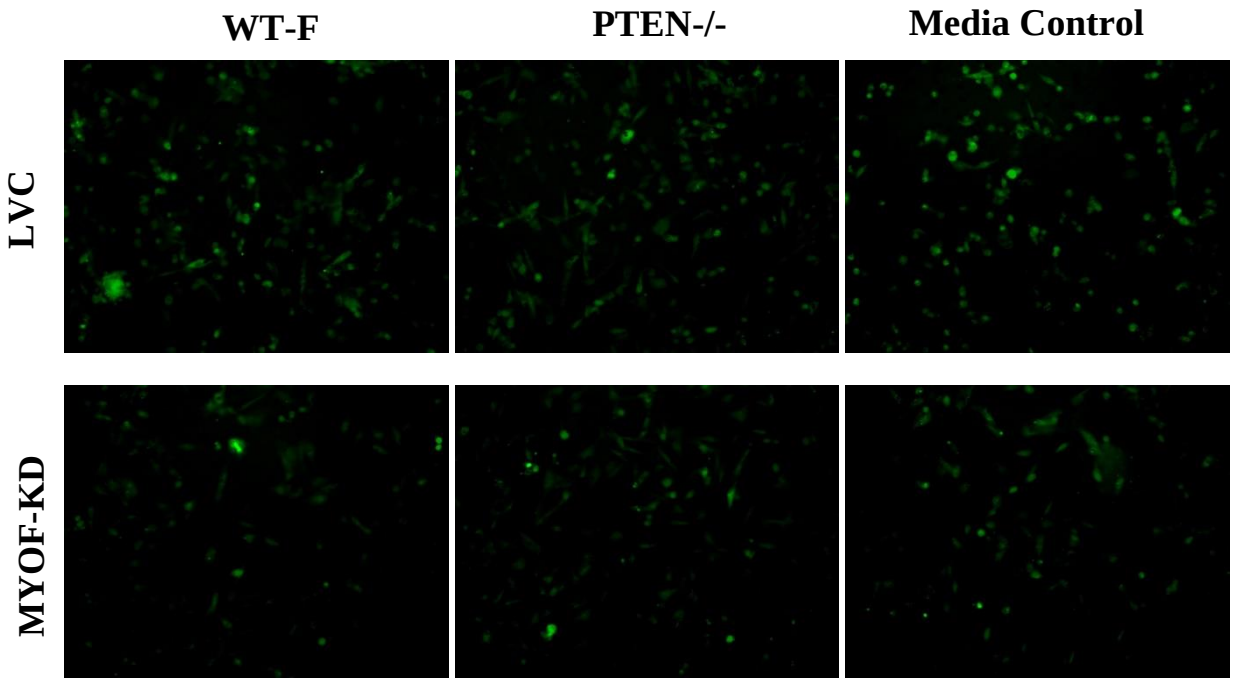

Figure 3-13 Collagen Invasion Assay Fluorescent Images: Fluorescent images of invading cells in transwell dishes. There is visibly less invasion by MYOF-KD cells compared to LVC cells. However, it is difficult to determine frim the images differences in invasion among fibroblast type.

In order to more accurately analyze the invasion data, the number of invading cells was quantified and statistical analysis was conducted. The data was normally distributed and the mean number of invading cells ± standard deviation are reported in Figs 3-14 and 3-15. As predicted from the images, MYOF-KD cells migrated significantly less than LVC MDA-MB-231 cells regardless of the fibroblast type used in the invasion assay (Fig 3-14). In fact, there were no significant differences among MYOF-KD cell invasion for all fibroblast types and as hypothesized, PTEN-/- fibroblasts generated the largest invasion (Fig 3-15). There was also no statistical difference between the invasion of the LVC and MYOF-KD cells when in the presence of the media control and WT-F stromal fibroblasts, indicating that WT-F stromal fibroblasts do not contribute to the enhanced migratory properties of a tumor microenvironment like PTEN-/- fibroblasts do. These results indicate that as hypothesized, not only are the migratory properties of

MDA-MB-231 cells reduced on 2D platforms when MYOF is knocked down, but their migration and invasion is also reduced in a 3D environment more similar to in vivo conditions. The enhanced invasion due to PTEN-/- fibroblasts is likely due to mechanical remodeling of the matrix, excretion of a soluble factor, or a combination of both.

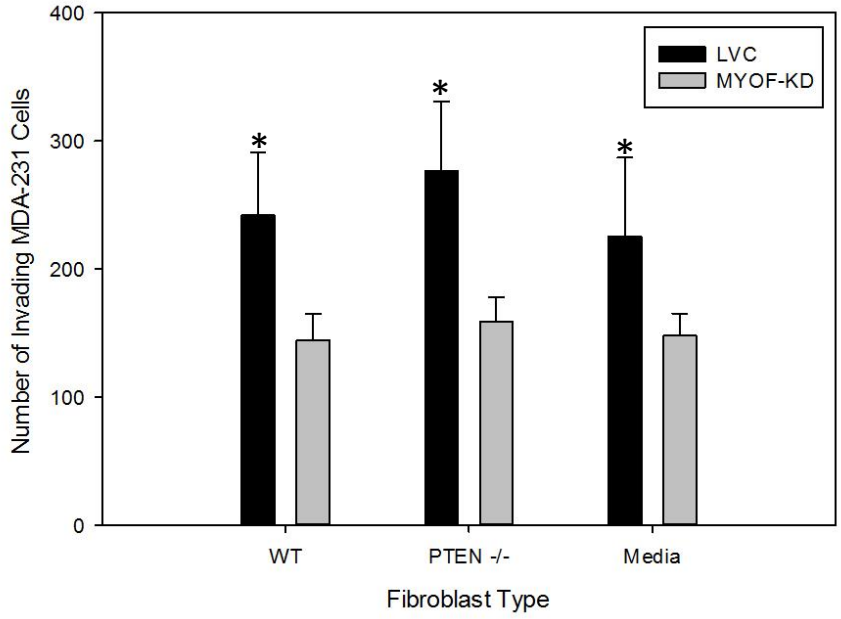

Figure 3-14 Collagen Invasion Assay LVC and MYOF-KD Comparison: Quantification of number of invading MDA-MB-231 cells.* indicates statistical significant ($p<.001$) differences in invasion of MYOF-KD and LVC MDA-MB-231 cells within same fibroblast type

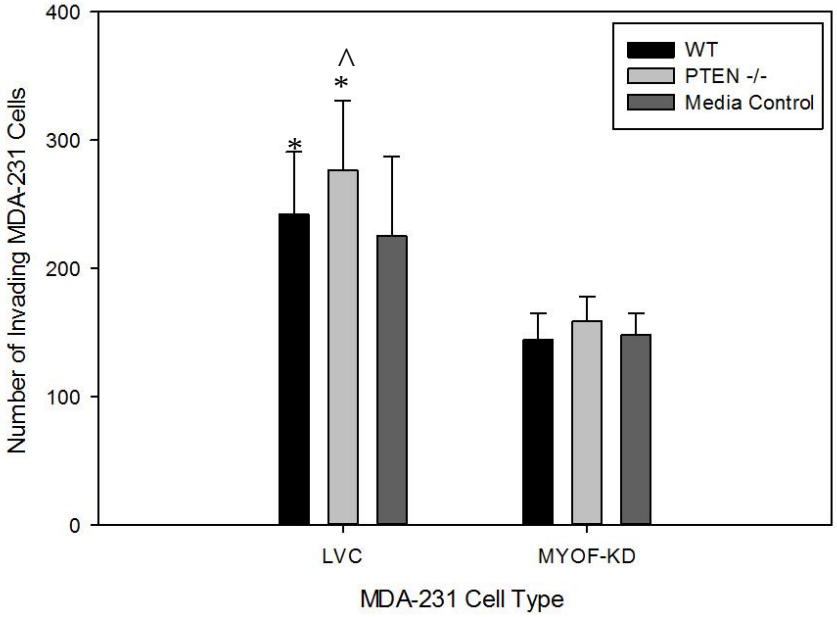

Figure 3-15 Collagen Invasion Assay Fibroblast Type Comparison: Quantification of number of invading MDA-MB-231 cells.* indicates statistical significant (p=.001) differences in invasion of LVC cells between PTEN-/- fibroblasts and the media control. ^ indicates statistical significant (p<.05) differences in invasion of LVC cells between PTEN-/- fibroblasts and the WT-F fibroblasts.

Conditioned media experiments were conducted to determine if a soluble factor excreted by the stromal fibroblasts is impacting the invasion of the MDA-MB-231 cells. Fluorescent images of invading cells were taken 24 hours after treatment with conditioned media (Fig 3-16). It is clear that the media control (serum free media) for both LVC and MYOF-KD cells had the least migration which could indicate that there is some factor in the conditioned media that leads to increased invasion.

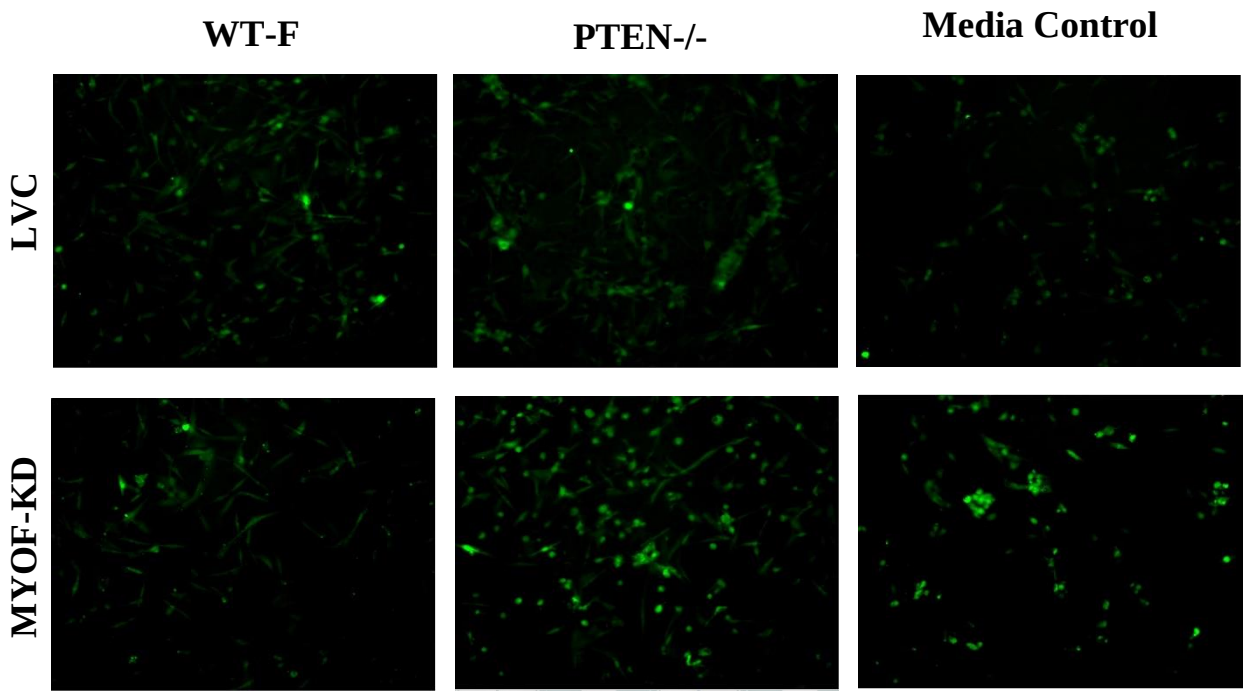

Figure 3-16 Conditioned Media Invasion Assay Florescent Images: There is visibly less invasion in both LVC and MYOF-KD cells for the serum free media control than in WT-F and PTEN-/- conditioned media.

In order to more accurately analyze the invasion data, the number of invading cells was quantified and statistical analysis was conducted. The data was normally distributed and the mean number of invading cells ± standard deviation are reported in the graphs below. Unlike the collagen invasion assays conducted with fibroblasts in the gels, MYOF-KD cells did not invade less for all fibroblast conditioned media type (Fig 3-17). There was no statistically significant difference in invasion between LVC and MYOF-KD cells for PTEN-/- conditioned media or the serum free media control. Between fibroblast type, unlike the collagen invasion assay, PTEN-/- fibroblast conditioned media resulted in the greatest invasion for MYOF-KD cells and not LVC cells (Fig 3-18). LVC cell invasion was not significantly different for WT-F and PTEN-/- conditioned media. In addition, WT-F conditioned media lead to significantly greater invasion of LVC cells than the serum free media control which was also not seen in the collagen invasion assay with fibroblasts.

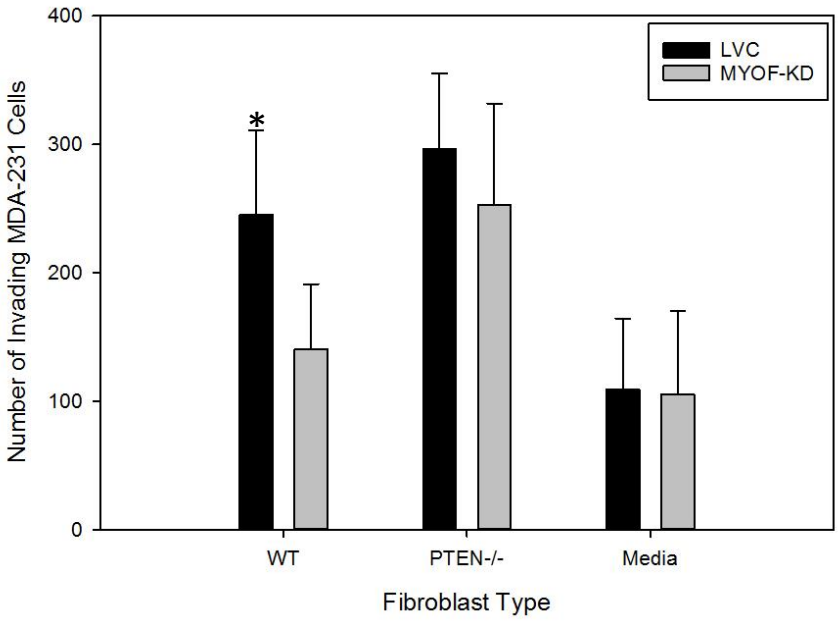

Figure 3-17 Conditioned Media LVC and MYOF-KD Comparison: Quantification of number of invading MDA-MB-231 cells. * indicates statistical significant (p<.001) differences in invasion of MYOF-KD and LVC MDA-MB-231 cells within same fibroblast conditioned media type

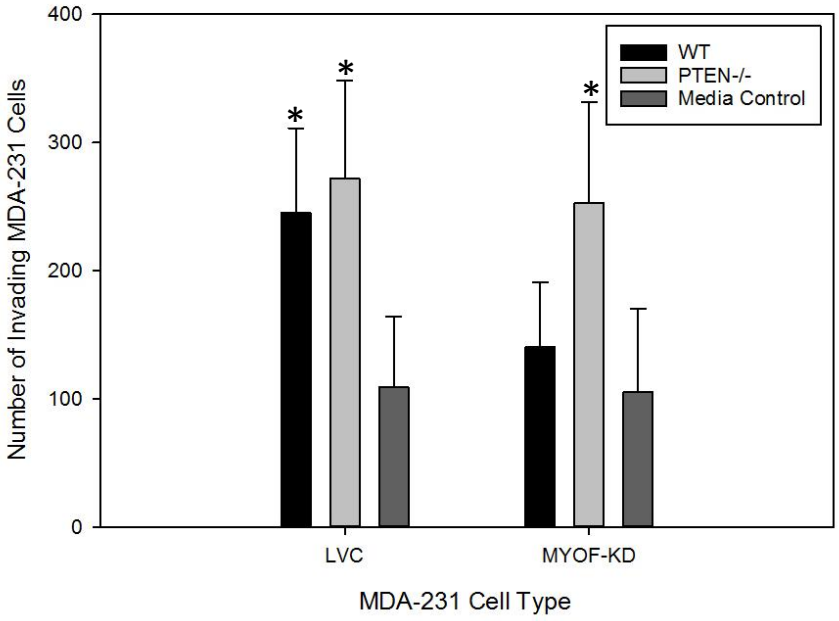

Figure 3-18 Conditioned Media Invasion Assay Fibroblast Comparison: Quantification of number of invading MDA-MB-231 cells. * indicates statistical significant (p<.001) differences in invasion of either LVC or MYOF-KD cells between PTEN-/- fibroblast conditioned media and the serum free media control.

4. Discussion and Conclusion

4.1 The Effect of Substrate Stiffness on MDA-MB-231 Cell Migration

Low stiffness polyacrylamide gels were used to mimic in vivo body stiffness to determine if, as previous studies on TCP found, that the knockdown of MYOF is effective in reducing the invasive properties of MDA-MB-231 cells (Volakis, 2014). On both the 2 and 20 kPa gel, distance migrated was found to be significantly reduced by the 24 hour time point in MYOF-KD cells compared to LVC cells. However, there was no significant difference in migration between the two MDA-MB-231 cell types on the 0.5 kPa gel. It is hypothesized that this is occurring because the lower stiffness may be reverting the LVC cells from their mesenchymal form to a more epithelial cell type, or mesenchymal to epithelial transition (MET). The figures on the 0.5 kPa gels (Figures 3-1 and 3-2) show both the LVC and MYOF-KD cells to appear more rounded and less elongated than on other gels which is typical of epithelial cell morphology. It is important to note that this change in shape could simply be due to decreased adhesion forces the cells are able to exert on the 0.5 kPa gel because it is so soft. Previous studies in vitro have found that more rigid substrates produce higher traction forces that promote epithelial cell migration and spreading (Rooij, 2005). In order to truly determine if the 0.5 kPa gel is inducing the LVC cells to go through MET, EMT cell markers will need to be tested for.

For the limited results available now, western blot data is consistent with similar experiments conducted on tissue culture plastic (Volakis, 2014). This indicates that even on stiffness more similar to normal and tumor mammary tissue in vivo, the knockdown of MYOF is likely still effective in causing tumor cells to revert back to an epithelial phenotype. In the future, additional western blots will be done and levels of the EMT markers E-cad and vimentin will be

quantified to determine if substrate stiffness has an effect on EMT/MET regulation on LVC and MYOF-KD MDA-MB-231 cells.

4.2 Effect of Stromal Fibroblasts on MDA-MB-231 Cell Invasion

A collagen invasion assay was conducted to determine the effect of cancer associated fibroblasts (CAFs), PTEN-/-, on MDA-MB-231 cell invasion. Although the results indicated that PTEN-/- stromal fibroblasts increase the invasive properties of LVC MDA-MB-231 cells, additional experiments need to be conducted to determine the mechanism behind this increase in invasion in order to develop possible treatment methods. During tumor formation and metastasis, activated stromal fibroblasts both remodel the matrix and excrete soluble factors which can increase the migratory properties of cells (Shiga, 2015). It is unknown by which mechanism the PTEN-/- fibroblasts increase the migratory behavior of LVC cells, and determining this is important for a better understanding of the breast cancer tumor microenvironment.

A better understanding of the mechanism by which the PTEN-/- fibroblasts increase the invasive capacity of LVC cells will guide the development of potential preventative treatments. For instance, if it is a soluble factor, an inhibitor could be designed to either target its excretion from fibroblasts or reception into epithelial cells. However, if it is due to mechanical remodeling, focus could be placed on developing ways to prevent collagen deposition of PTEN-/- fibroblasts, which could help to reduce matrix remodeling. It is possible that it is a combination of both factors, and therefore a synthesized inhibition method would need to be developed.

One potential prevention method that appeared to reduce invasive capacity of MDA-MB-231 cells is the knockdown of MYOF. Because the MYOF-KD cells did not experience an increase in invasion regardless of the fibroblast used in the invasion assay, this indicates that they may not be as susceptible to the changes induced by PTEN-/- fibroblasts. It is important to note

that their invasion was not nonexistent, thus having a better understanding of how PTEN-/- fibroblasts drive invasion of MDA-MB-231 cells will only help to further prevent it.

A conditioned media invasion assay was conducted to determine the possible presence of soluble factors being excreted by the stromal fibroblasts that could be increasing the invasive capacity of MDA-MB-231 cells. Serum free media was used to ensure that growth factors typically in DMEM with FBS were not impacting the invasion of the MDA-MB-231 cells. The LVC cells treated with WT-F fibroblast conditioned media also experienced greater invasion than the serum free media control. However, the MYOF-KD cells did not experience the same increase in invasion. This further validates the benefit of knocking down MYOF in preventing metastatic behavior of tumor epithelial cells. The results indicate the possible presence of soluble factors, most notably in the fact that MYOF-KD cell invasion was significantly increased when treated with PTEN-/- fibroblast conditioned media than with WT-F fibroblast conditioned media or serum free media. In addition, LVC cells experienced significantly greater invasion than the serum free media control when treated with PTEN-/- fibroblast conditioned media. This result likely indicates soluble factors present in the PTEN-/-conditioned media that both LVC and MYOF-KD cells are susceptible to. Determining this soluble factor will be important in the development of potential treatments.

These results highlights the need to have a better understanding of the potential soluble factors stromal fibroblasts may be secreting. Previous studies have found that the soluble factors TGF-β and TNF-α can drive mammary epithelial cell invasion (Bhome, 2015). In addition, in a study of stromal mammary fibroblasts the cytokines granulocyte chemotactic peptide (GCP)-2, interleukin (IL)-6, IL-8, tissue inhibitor of metalloproteinases (TIMPs), growth-related oncogene (GRO), and monocyte chemoattractant protein (MCP)-1 were all found to be upregulated in culture

(Olsen, 2010). Future experiments will seek to determine possible soluble factors secreted by the WT-F and PTEN-/- fibroblasts that interact with the MDA-MB-231 cells.

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